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DOI: <https://doi.org/10.1007/s00265-005-0152-2>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-155816>

Journal Article

Published Version

Originally published at:

Tobler, Michael; Plath, Martin; Burmeister, Heike; Schlupp, Ingo (2006). Black spots and female association preferences in a sexual/asexual mating complex (*Poecilia*, Poeciliidae, Teleostei). *Behavioral Ecology and Sociobiology*, 60(2):159-165.

DOI: <https://doi.org/10.1007/s00265-005-0152-2>

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Received: 6 August 2005 / Revised: 15 October 2005 / Accepted: 18 December 2005 / Published online: 1 March 2006
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Abstract We investigated whether female association preferences for males are influenced by black spot disease (BSD), a parasite induced change of the host phenotype. We compared three different species of fish: a gynogenetic hybrid species, *Poecilia formosa* (amazon molly) and two sexual species (*Poecilia latipinna* and *Poecilia mexicana*), which were involved in the natural hybridisation leading to the amazon molly. Contrary to their sexual relatives, asexual amazon mollies significantly avoided images of males infected with black spot disease. We propose that amazon molly females have direct fitness benefits from choosing healthy males. The adaptive significance of the preference for BSD-uninfected males in the asexual amazon molly is yet unclear but may involve avoidance of predation or parasite infection as well as increased sperm availability.

Keywords Black spot disease · Parasites · Red Queen hypothesis · Sexual selection · *Uvulifer* · Video playback

Communicated by K. Lindström

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Introduction

The effects of parasites on the mating success of their host have received considerable attention since Hamilton and Zuk's (1982) theory about the role of parasites in sexual selection was proposed. Elaborated male ornaments may serve as an indicator for low parasite load (Milinski and Bakker 1990; Lindström and Lundström 2000; Taskinen and Kortet 2002). Mating with unparasitised males is thought to provide direct fitness benefits if parasites can be transmitted and indirect benefits if parasite resistance is heritable (Hamilton and Zuk 1982; Read 1988; Barber et al. 2001). Experimental evidence for the avoidance of parasitised males by females comes from a variety of taxa including several fishes (Kennedy et al. 1987; Milinski and Bakker 1990; Houde and Torio 1992; Rosenqvist and Johansson 1995; Taylor et al. 1998; Barber et al. 2001; Plath 2004), but the evidence is not unequivocal (Barber 2002).

Parasites often change the hosts' phenotype indirectly: ornamental traits are less expressed. However, parasites can also alter the male phenotype directly. If females can perceive such direct changes, their mate choice decisions may be influenced (Endler and Lyles 1989). In the present study, we examined female choice relative to an indirectly transmitted parasite that directly affects the hosts' phenotype, black spot disease (BSD). BSD is caused by the metacercariae of a trematode (*Uvulifer* sp.), which induces the production of black spots on the body surface of the fish (Bush et al. 2001). It is unknown so far if BSD infection affects the expression of ornaments as assumed by Hamilton and Zuk (1982), but the visible black cysts clearly have a direct effect on the infected males' phenotype (Endler and Lyles 1989).

Among other fish species, *Uvulifer* sp. infects the members of a reproductive complex consisting of two sexual and a gynogenetic species of live-bearing fishes (Poeciliidae): The asexual *Poecilia formosa* (amazon molly) and its sexual relatives *P. latipinna* (sailfin molly) and *P. mexicana* (Atlantic molly), which have been involved in the natural hybridisation leading to the amazon

molly (Schartl et al. 1995). Most importantly, the amazon molly lives in the same microhabitats as its sperm donors and forms mixed groups with at least one of the parental species (Hubbs 1964; Balsano et al. 1989; Schlupp et al. 2002). Therefore, the exposure to parasites is very similar (Tobler et al. 2005).

Contrary to sexual females, amazon mollies require sperm only to trigger embryogenesis (reviewed in Schlupp 2005), therefore it seems that most mating preferences are not currently under selection because indirect benefits such as good genes are not possible because there is no fertilisation. For example, Marler and Ryan (1997) documented a preference for larger males in *P. formosa*, although the most obvious direct benefit, the triggering of embryogenesis, did not vary with the size of the sperm donor. The persistence of this sexual preference has been attributed to an evolutionary time lag (Marler and Ryan 1997). This raises the question on how females of this species respond to obviously infected males. Three different theoretical frameworks, which make different predictions, can be contrasted:

- (1) Sexual females should avoid infected males if BSD indicates males of poor genetic quality (Hamilton and Zuk 1982). If such indirect benefits for choosing females are relevant, amazon mollies were not predicted to show a preference because any information relative to “good genes” is irrelevant to them.
- (2) Sexual females should prefer spotted males if surviving with BSD indicates high immunocompetence (Zahavi 1975). In this case again, *P. formosa* is not expected to show a preference.
- (3) Direct benefits, such as the avoidance of parasite transmission, may be relevant for the females (Able 1996). BSD, although not directly transmitted itself, may be an indicator for males with poor general condition carrying other contagious pathogens (Lane and Morris 2000). In this case, the preference for healthy males may be identical in asexuals and sexuals, but it might be even stronger in the asexuals due to their higher susceptibility to parasites: According to the Red Queen hypothesis, sexual reproduction is an adaptation against parasites (Seeger and Hamilton 1988). Clonal species are supposed to suffer from high parasite loads (Hamilton 1980; Lively 1987; Seeger and Hamilton 1988; Hamilton et al. 1990; Lively et al. 1990; Ladle 1992; Hakoyama et al. 2001). Recent studies failed to detect significant differences in parasitisation between sexual and asexual mollies (Tobler and Schlupp 2005; Tobler et al. 2005), but the patterns of parasitism in the reproductive complex of *P. formosa* are yet poorly understood, and asexuals may generally have a higher susceptibility to parasite infections.

Specifically, we examined how females of the three species of mollies respond to males showing signs of parasite-induced black pigmentation based on visual information. A traditional approach to this question

would be examining female preferences using infected and uninfected stimuli (e.g., Houde and Torio 1992; Rosenqvist and Johansson 1995; Plath 2004). However, parasitised males may be in worse general condition than healthy males, and thus, might behave differently (Tobler, unpublished data). As a result, females might respond to the altered behaviour and not to the black spots under consideration. Video playback techniques allow bypassing of this problem and provide behaviourally uniform stimuli differing only in the presence of black spots. Video playback has been a useful tool for behavioural studies of numerous taxa including fishes (McKinnon 1995; Rowland et al. 1995; Rosenthal et al. 1996; Rosenthal and Evans 1998; Nicoletto and Kodric-Brown 1999; Rosenthal 1999; Oliveira et al. 2000) and especially mollies (Körner et al. 1999; Landmann et al. 1999; Gonçalves et al. 2000; Plath et al. 2003; Witte and Ueding 2003; Witte and Klink 2005). A critical review and recommendations on how to use video playback has been provided by Oliveira et al. (2000).

Materials and methods

Study organisms: the parasite

BSD in mollies is caused by metacercariae of a trematode (*Uvulifer* spp., Diplostomatidae) with a complex life cycle. Thus, it is evident that BSD cannot be transmitted directly from fish to fish. Kingfishers (*Ceryle* sp.) are the end hosts and pulmonate snails (Planorbidae) the first intermediate hosts in which the parasite multiplies asexually, and free-swimming cercariae are produced. These infect fish as second intermediate hosts by penetrating their skin and transforming into encysted metacercariae (Lane and Morris 2000). The metacercariae provoke the production of a fibrous capsule of host tissue around the parasite, which is followed by the migration of melanocytes into the cyst's wall creating the characteristic appearance of the black spot (Spellman and Johnson 1987; Bush et al. 2001). This reaction of the host is assumed to be costly: The penetration through the skin causes mechanical damage, which also facilitates secondary infections with other pathogens (Lane and Morris 2000; Bush et al. 2001). Until the parasite becomes encapsulated, the host's metabolic demand increases significantly, and thus, energy reserves required for the survival in harsh environmental conditions may decline (Lemly and Esch 1984; Lane and Morris 2000; Bush et al. 2001). Further costs after encystation may involve a higher predation risk due to an increased conspicuousness, reduced response to predators and reduced shoaling behaviour (Krause and Godin 1994, 1996). Besides diplostomatid trematodes, no other pathogens are known to induce black-spotted phenotypes. Black spots in poeciliids, however, are not always induced by parasites. In guppies, *P. reticulata*, male black pigmentation is thought to serve as visual amplifier (Brooks 1996). In several mollies (*Poecilia*), swordtails (*Xiphophorus*) and mosquito fish (*Gambusia*), black spotted phenotypes exist (Meyer et al. 1985). “Natural” black spots, however, are absent in the molly

populations studied here. Parasite induced black spots are very conspicuous—at least to the human eye. They can easily be differentiated from other patterns formed by melanin-containing cells (micro- and macro-melanophores) by their size, intensity and their random distribution on the body of the fish (Tobler, unpublished data). In many cases, non-parasite induced black spots are found in defined places (i.e. on the caudal peduncle in *Xiphophorus maculatus*; Scharl 1995).

Study organisms: fishes

P. formosa, the amazon molly, is an all-female, ameiotic and clonal species that originated through natural hybridisation of a *P. mexicana*-like female and a *P. latipinna*-like male around 100,000 generations ago (Avisé et al. 1991; Scharl et al. 1995). Amazon mollies are gynogenetic, and thus, rely on sperm to trigger embryogenesis, but normally no paternal DNA is transmitted so that inheritance is clonal. In the northern part of its distribution (southeastern Texas), *P. formosa* coexists with *P. latipinna*, the males of which serve as sperm donors. In the southern part (northern Mexico), the species coexists with and relies on the sperm of *P. mexicana* (Schlupp et al. 1998; Schlupp et al. 2002).

We used *P. formosa* and *P. latipinna* from a population in the Guadalupe River system (San Marcos River and Comal River, Central Texas, USA). Both species were introduced into Central Texas and established thriving populations (Brown 1953). *P. latipinna* was introduced from Louisiana in the 1940s, *P. formosa* from Brownsville (South Texas) in the 1950s (Schlupp et al. 2002). While *P. formosa* has a low clonal variability based on allozyme data (Turner 1982; Turner et al. 1983), *P. latipinna* from the Guadalupe River system are known to have a high degree of heterozygosity (Simanek 1978). Furthermore, we used *P. mexicana* and *P. formosa* from the Rio Purificacion drainage system (Tamaulipas, Mexico), where both species naturally coexist.

Fish were maintained in aerated and filtered 50–200 l tanks at 24–30°C at the Biozentrum Grindel in Hamburg as randomly outbred laboratory populations. Fish were daily-fed *ad libitum* flake food and *Artemia* nauplii. The three species were kept separated. Females were not separated from males prior to testing. All females tested were naïve with respect to BSD-infected males.

P. latipinna males used to produce the computer animations were collected in the San Marcos River in autumn of 2003 and transported to the laboratory of the University of Texas in Austin. The *P. mexicana* male came from a semi-natural outdoor breeding pond at the University of Texas Brackenridge Field Laboratory. No BSD-infected *P. mexicana* males were available at the time of the study.

Video animations

Three different pairs of animations were generated: (1) a healthy male of *P. latipinna* and its identical copy with artificially added black spots (virtual size of the males 46 mm, 12 black spots on the artificially infected male), (2) a BSD-infected male of *P. latipinna* and its identical copy with artificially removed black spots (virtual size of the males 40 mm, 3 black spots on the naturally infected male) and (3) a healthy male of *P. mexicana* and its identical copy with artificially added black spots (virtual size of the males 46 mm, 12 black spots on the artificially infected male). The sizes of the males as well as the numbers of black spots (trematode cysts) were within the natural ranges for these species. By adding 12 spots (animations 1 and 3), we attempted to generate a heavily infected male contrasting to animation 2, where the intensity of infection was around the natural mean (Tobler, unpublished data).

Animation pairs 1 and 2 were used to test *P. latipinna* females as well as *P. formosa* that live in sympatry with *P. latipinna*. The videos of animation pair 1 and 2 were never mixed; thus, either animation pair 1 or 2 was presented to an individual female. Animation pair 3 was used to test *P. mexicana* females and *P. formosa* that live in sympatry with *P. mexicana*.

To produce the video animations, digital images of each healthy and BSD-infected *P. latipinna* male were taken while a male was swimming in a small tank using a Fuji Finepix S602Z digital camera. From the resulting images, the males were cut out using “Adobe Photoshop 7.0”. For animation pair 1, three infected individuals were photographed in the field. One black spot of each individual was cut out digitally from these images and each spot was then pasted four times onto the body of the healthy male. Using the same technique, the black spots of the BSD infected male were covered up with scales for animation pair 2. For animation 3, we pasted black spots onto the body of a healthy male of *P. mexicana* as described for animation 1. As a result, three pairs of animation showed two identical males differing only in the presence or absence of black spots. Differences in the response to the animation by the tested females can therefore be attributed to the trait under study, black spots.

The resulting images were then animated using “Adobe Premiere 4.2”. A straight movement of the pictures from left to right and right to left was generated in front of a uniform grey background. The animations were 14 s long: twice 6 s for the distance of 28.5 cm on the screen forth and back, each followed by an invisible turn of 1 s. Images were transformed to greyscale and AVI movies were generated for playback. Transformation to greyscale allowed us to exclude the potentially confounding effects of an interaction between black and yellow pigmentation (Brooks 1996).

Simultaneous playback was performed using two identical computer monitors (Belinea 10 30 40) with a Matrox Millenium G400 Dual Head graphic card. The monitor refresh rate was 85 Hz. The AVI-films were run using “Windows Media Player” in infinite loops.

Preference tests

The monitors were placed on either sides of a test tank (60×32×32 cm). Water level was maintained at 24 cm, which was also the height of the monitors. A thin layer of gravel covered the bottom. The water temperature was 25°C. The tank was visually divided into three sections by markings drawn on the front: a central neutral zone (25 cm) and two preference zones near the monitors (each 17.5 cm).

Before a test, the video playbacks were started. Then a test female, randomly selected from a stock tank, was placed into a transparent Plexiglas cylinder (8.5 cm in diameter) in the middle of the neutral zone. The female was allowed to acclimate until she moved freely (2 to 5 min). Then, the cylinder was carefully removed and an observation period of 5 min followed. We recorded the time spent in each preference zone. To detect side biases, the female was immediately placed into the cylinder again, the video playbacks were switched and the measurement was repeated. After the test, the standard length of the female was measured to the closest millimetre. The females were then transferred into another tank so that each was only tested once.

We decided a priori to exclude side biases (more than 80% of time during both parts of a trial in only one preference zone) and trials with low response (less than 50% of the time inside the preference zones). We assumed such females not to be motivated to choose (Landmann et al. 1999). Side biases occurred in 48 cases (out of 199 trials total). No trial was excluded due to low response.

Statistical analysis

The relative time spent in each preference zone was calculated, and the data were arcsin-transformed to achieve normal distribution. To test for female association preferences within populations, the relative time spent near either type of stimulus male was compared using paired *t* tests.

To test for population differences in female preferences, we calculated the strength of preference $[(\text{time}_{\text{healthy}} - \text{time}_{\text{BSD}}) / (\text{time}_{\text{healthy}} + \text{time}_{\text{BSD}})]$ and compared it among populations using analysis of covariance (ANCOVA) with female standard length as a covariate. For post hoc contrasts, Fisher's protected least significant difference was used. For the tests with *P. latipinna* and their sympatric *P. formosa*, we used two video animations differing in the template used. We tested if the two different types of animation had an influence on the strength of preference using two-sample *t* tests. Alpha levels were corrected according to the number of multiple compar-

isons using Bonferroni adjustments ($\alpha' = 0.05/\text{number of multiple comparisons}$).

In our study, the independent unit of replication is individual females. We are comparing female association preferences among three different species. To measure the females' responses, we used pairs of video-stimuli that were identical except for the presence or absence of black spots. For technical reasons, we were limited to only three pairs of video stimuli. In a study using live stimuli, more stimulus exemplars would have been used as control for traits that co-vary with the trait under consideration (McGregor 2000). By using the same individual to create the control and manipulated animation, the animations are the same except for the manipulation. Thus, co-variation in any other trait does not exist and does not need to be controlled (see also Rosenthal and Evans 1998; Morris et al. 2003, 2005).

A potential problem is an interaction between the trait under consideration and any other trait crucial for the behavioural response of the test individual. For example, females may respond differently to infected males depending on male size or the relative size of the male's dorsal fin. The two *P. latipinna* males used to create the video animations were selected from the extreme ends of a continuum (small, non-ornamented, and non-courting on the one side to large, ornamented and courting on the other; Parzefall 1969). Because female responses do not differ between the two *P. latipinna* animations, such an interaction seems very unlikely, but it cannot be excluded statistically.

Results

Female preferences

Like in previous studies, the females clearly responded to the videos. They oriented towards the animated images and often followed them over longer periods of time. We successfully tested 151 females (Table 1).

Table 1 The number (*n*) of successfully tested females with each animation pair, and the standard length (SL mean±SD) of the tested females

Species	Population	SL [mm]	Tested with animation pair	<i>n</i>
<i>P. latipinna</i>		35.9±6.7	(1)	46
			(2)	20
<i>P. mexicana</i>		35.3±4.9	(3)	22
<i>P. formosa</i>	Sympatric with <i>P. latipinna</i>	38.4±5.5	(1)	20
			(2)	19
<i>P. formosa</i>	Sympatric with <i>P. mexicana</i>	39.1±6.3	(3)	24

The asexual *P. formosa* showed a significant preference for the unspotted male both with *P. latipinna* ($t_{38}=-3.27$; $P=0.002$; $\alpha'=0.017$) and *P. mexicana* animations ($t_{23}=-2.67$; $P=0.014$; $\alpha'=0.025$; Fig. 1). *P. latipinna* and *P. mexicana* females showed no detectable preference (*P. latipinna* $t_{65}=0.10$; $P=0.92$; $\alpha'=0.017$; *P. mexicana* $t_{21}=-0.18$; $P=0.85$; $\alpha'=0.025$; Fig. 1).

Comparison between populations and animations

Because the interaction terms were not significant in the ANCOVA, only the main effects were analysed. The covariate 'standard length' had no significant influence on the strength of preference ($F_{1,147}=0.38$; $P=0.54$). The strength of preference differed significantly among the populations ($F_{3,147}=3.60$; $P=0.015$; $\alpha'=0.017$). A post hoc test revealed that *P. formosa* differed significantly from sympatric *P. latipinna* females ($P=0.002$). *P. formosa* sympatric with *P. mexicana* differed neither significantly from the corresponding *P. mexicana* females ($P=0.10$), nor from the other *P. formosa* population ($P=0.76$).

There was no significant difference in the strength of preference between the two pairs of *P. latipinna* animations used (*P. latipinna* $t_{64}=-0.47$; $P=0.64$; $\alpha'=0.017$; *P. formosa* $t_{37}=0.29$; $P=0.78$; $\alpha'=0.017$), indicating that females do not base their decisions on whether we used a healthy or infected male as template and on the intensity of infection, respectively.

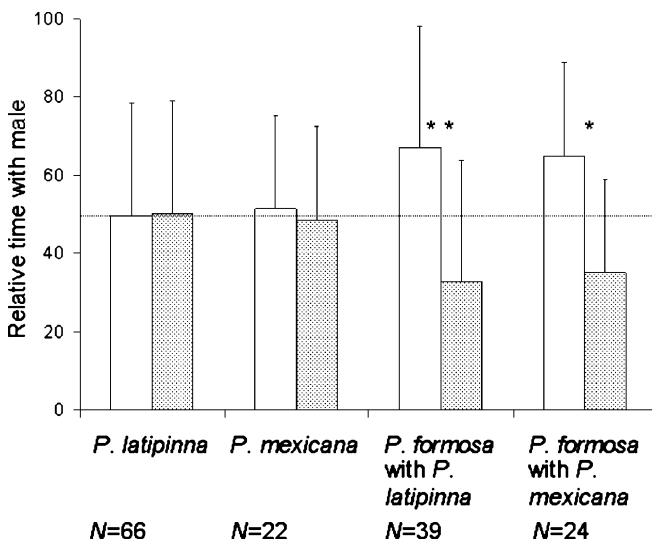


Fig. 1 Female preferences relative to BSD infection: The relative time (mean \pm SD) spent near a monitor showing a healthy male (open bars) or a male infected with BSD (shaded bars). The sexual species *P. latipinna* and *P. mexicana* did not show a preference. In contrast, a preference for uninfected males was found in both asexual *P. formosa* populations (paired *t* test, two-tailed; * $P<0.05$, ** $P<0.01$)

Discussion

Given a choice between an apparently healthy and an apparently BSD-infected male, females of the asexual amazon molly preferred to associate with the healthy male. In contrast, the females of two sexual relatives showed no detectable preference. It is unlikely that the difference between asexual and sexual females is due to a reduced motivation or lower response to video animations in the latter because sexual females have independently been shown to respond to video animations (Witte and Ueding 2003; Witte and Klink 2005; Schlupp, unpublished data). In the present study, sexual females spent the same amount of time in the preference zones responding to the video animations as the asexual females (analysis not shown), but a preference is only present in asexual females.

This study demonstrates that visual information alone is sufficient to elicit female responses. Amazon mollies clearly respond to the phenotypic change induced by the parasite per se. These results are consistent with hypothesis 3, in which direct benefits are assumed to maintain the female preference. Our results contrast with the response to a novel male trait, an orange tumour, where females of both sexual species preferred males with the unusual trait and the asexual species showed no preference (Schlupp et al. 1999). Our result that amazon mollies show an association preference for healthy males which is absent in the parental hosts species is interesting for at least two reasons:

- (1) The association preference detected in the amazon molly is apparently absent in both parental species and may be a derived trait, which has evolved in the asexual hybrid. This is unexpected because the evolutionary potential of an ameiotic, clonal species is usually considered to be very limited. Other preferences in the amazon molly, like the one for large male body size have been viewed as ancestral, because they also occur in the parental species (Ryan et al. 1996; Marler and Ryan 1997). Alternatively, the difference between the sexuals and the asexuals may be explained by regressive evolution in the two parental species.
- (2) Our finding raises the question why asexuals show a preference, while the sexual species do not. If sexuals do not use the information provided by infected males, why do asexuals? BSD-infected individuals have been hypothesised to be avoided as shoal mates because they may attract predation (Krause and Godin 1994, 1996). This idea is consistent with the reported avoidance found in amazon mollies, but fails to explain the behaviour of the sexual females, which show equal tendencies to form shoals.

Because our finding was surprising but potentially relevant, we suggest several mechanisms that could lead to this result; however, none of these have been tested so far: We suggest that the costs of interacting with a parasitised male differ for asexuals and sexuals. BSD is not directly transmitted, but may indicate a male's

susceptibility to infections and a debilitated immune system. BSD is known to reduce energy reserves of infected fish (Lemly and Esch 1984; Bush et al. 2001), which is likely to negatively affect the immune system, and thus, facilitates secondary infections with other parasites and diseases (Lane and Morris 2000). Hence, BSD may be correlated with higher rates of infestations with other parasites. A correlation analysis between BSD infection and 11 other eukaryotic parasite species from 110 *P. formosa* and 66 *P. latipinna* collected in four populations did not reveal such a pattern (Tobler, unpublished data), but data including more important contagious pathogens, namely, fungi, bacteria and viruses, are lacking so far.

According to the Red Queen hypothesis, the production of new genotypes and the advantage over parasites adapting quickly to common host genotypes, is the main benefit of sex and recombination. While the cost of not avoiding BSD-infected males may be trivial for sexuals, a clonal species like the amazon molly may be much more vulnerable and may benefit strongly from avoiding any kind of infection risk. Contrary to other studies on asexual fish (e.g., *Poeciliopsis*; Lively et al. 1990 and *Carassius*; Hakoyama et al. 2001), a higher parasite load in the asexual amazon molly could not be detected in a preliminary study (Tobler and Schlupp 2005; Tobler et al. 2005). However, a lack of difference in the parasite loads between sexuals and asexuals does not necessarily indicate that Red Queen mechanisms do not play a role in this system. Behavioural adaptations to avoid parasite infections may rather be a crucial factor to explain the strikingly even degree of parasitisation compared to the sexual sailfin molly. Possibly, differences in parasitisation are much smaller than expected by the Red Queen hypothesis because the genetically more susceptible asexuals have evolved behavioural counter-adaptations like the preference reported in this study.

Besides increased infection risk, sperm availability may be a crucial factor: Male *P. mexicana* not only prefer to mate with conspecific females (as do *P. latipinna* males; Schlupp et al. 1991), but they also allocate significantly less sperm to asexual compared to sexual females (Schlupp and Plath 2005). Hubbs (1964) found that a higher proportion of oocytes is not developing in *P. formosa* compared to *P. latipinna*. Parasites may influence the quantity and/or quality of sperm of males (e.g. Liljedal et al. 1999). Better sperm availability in non-infected host males would be another plausible explanation for the observed preference in amazon mollies. On the contrary, sexual females are not assumed to suffer from sperm shortage because multiple mating is frequent in this mating system and conspecific males prefer them.

In summary, direct benefits best explain the observed differences in the female preference for males without parasite-induced black spots between sexual and asexual mollies. The adaptive significance of this preference in the

asexual amazon molly clearly deserves a more detailed examination in future studies.

Acknowledgements We thank Dustin Penn and Michael J. Ryan for critically reading an earlier draft of this paper. A. Taebel-Hellwig helped with the data collection; the Animal care teams of the Biozentrum Grindel; and University of Texas Brackenridge Field Laboratory helped with fish care. Financial support came from DFG (SCHL 344/13-1, I. S.), from the University of Hamburg (M. P.) and from the Swiss Academy of Sciences and the NWG St. Gallen (M. T.). The experiments reported in this paper comply with the current laws in Germany. The Mexican Government kindly issued permits to collect fish (Permiso de pesca de fomento numbers: 242-219, 276/36, 210696-213-03).

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